A MUCIN CLOT REACTION WITH SEA-URCHIN FERTILIZIN

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Introduction

Recent work on the fertilizins (the sperm-agglutinating constituents of egg water) of the eggs of sea-urchins and other animals has shown them to be of the nature of mucoproteins. Tyler and Fox (1939, 1940) showed that the fertilizins of Strongylocentrotus and of Megathura possess protein characteristics, but are of low nitrogen content. Similar evidence has been obtained with Arbacia fertilizin by Kuhn and Wallenfels (1940) and with Psammechinus fertilizin by Runnström, Tiselius, and Vasseur (1942). The latter workers also obtained a positive carbohydrate test. Tyler (1948) reported the presence of reducing sugars to the extent of about 15 per cent in hydrolyzed, purified preparations of Strongylocentrotus fertilizin and identified galactose as one of the constituents. As will be shown later in this paper, the present author has found hexosamine to be present in amounts equivalent to about 2 per cent of the original material. According to Runnström, Tiselius, and Vasseur (1942) and Tyler (1946) the sea-urchin fertilizins are of pronounced acidic character.

Acidic mucopolysaccharides are known (cf. Meyer, 1945, and Stacey, 1946) to co-precipitate with proteins upon acidification of the native fluid or of the neutral extracts in the form of "mucin clots," stringy or granular precipitates, depending upon the conditions of precipitation. This reaction is given, for example, by hyaluronic acid (Meyer and Palmer, 1936) and has been used in the assay of the enzyme hyaluronidase (McClean, 1943). It was of interest to determine whether or not fertilizin preparations would give the mucin clot reaction. As the work reported here shows, fertilizin preparations do give such a mucin clot reaction. A titration method, based upon this, was developed for these preparations, and comparisons made with their sperm-agglutinating activity in untreated condition, after dialysis, and after exposure to heat and to ultra-violet irradiation.

Materials and Methods

The sea-urchins *Lytechinus pictus, Strongylocentrotus purpuratus* and *S. franciscanus* were used in these experiments. Most of the work was done with *S. purpuratus.*

Two kinds of fertilizin preparations were employed. One, which will be termed "crude fertilizin," was prepared by acidifying a 20 per cent suspension of washed eggs to pH 3–3.5, removing the supernatant fluid after five or ten minutes and readjusting the pH of this solution to 7–7.5. The other, which will be termed "purified fertilizin," was further subjected to alkali precipitation, dialysis against 3.3 per cent acid saline (pH 3.5–4) and alcohol precipitation according to the method described by Tyler (1948). Material prepared in this manner has been

found to be electrophoretically homogeneous (Tyler, unpub.). For the various tests the solutions were made up in 3.3 per cent NaCl at a pH of about 7.

Sperm-agglutinating titer of the fertilizin preparations was determined using the drop method of preparing two-fold serial dilutions with sea water in Syracuse watch glasses. To two drops of each dilution of fertilizin solution one drop of a uniform sperm suspension, usually 1 per cent (calculated as 1 cc. dry sperm per 100 cc. sea water suspension), was added. The highest dilution in which agglutination is observable under the microscope gives the titer of the preparation.

Tests for univalent fertilizin were made according to the method described by Tyler (1941) and Metz (1942). Essentially, this method consists of first treating sperm with the solution containing univalent fertilizin, which does not agglutinate the sperm, and then adding an equal volume of strong normal fertilizin solution to the suspension of sperm. Failure of the sperm to be agglutinated by the normal fertilizin presumably indicates that the combining groups on the sperm surface have been occupied by univalent fertilizin groups and are no longer available to unite with the normal fertilizin. Univalent fertilizin was obtained by heating and by irradiation with ultra-violet light of normal fertilizin preparations (cf. Tyler, 1941, and Metz, 1942).

Bovine serum albumin prepared by the Armour laboratories was used in 1 per cent solution for the co-precipitation tests.

Hyaluronic acid was obtained from human umbilical cords according to the method described by McClean (1943), whereby the distilled water extract of acetone-dried, ground cords, extracted with 90 per cent acetic acid according to the method of Meyer and Palmer (1936), was precipitated with 1.25 volumes of cold, potassium acetate-saturated 95 per cent alcohol. The precipitate was washed with alcohol, acetone, and ether and dried over P_2O_5. The dry product was dissolved in distilled water as required; a solution of 0.1–0.2 per cent of the dry material was clear, viscous and did not form a precipitate upon the addition of acetic acid, but co-precipitated with serum albumin in the presence of acetic acid, forming a stringy clot. In higher dilutions the mixture of serum albumin, acetic acid and hyaluronic acid solution resulted in the formation of a fine precipitate or turbidity. The highest dilution in two-fold serial dilutions in which turbidity was perceptible by visual inspection was taken as the titer of the hyaluronic acid solution.[1]

Experiments and Observations

Co-precipitation of fertilizin with serum albumin in acid solution

A viscous solution of crude fertilizin of *Lytechinus pictus* was prepared as described above and combined with a 1 per cent solution of bovine serum albumin in 0.9 per cent NaCl and 2 N acetic acid according to the method described by McClean (1943) in the mucin clot test. With this solution a very large clot was formed similar in character and appearance to the clot formed by hyaluronic acid. Purified fertilizin preparations of *Strongylocentrotus purpuratus*, *S. franciscanus*, and *L. pictus* were tested in the same manner and in each case a clot or precipi-

[1] A quantity of pure potassium hyaluronate was later supplied to me by the Schering Corporation, through the courtesy of Dr. W. Alan Wright and Dr. Erwin Schwenk.

tate formed, depending upon the concentration of the solution. In Figure 1 a series of photographs of the mucin clot reaction of *S. purpuratus* fertilizin is shown, with the reaction given by hyaluronic acid for comparison.

By taking advantage of the biological activity of fertilizin, i.e., its sperm-agglutinating activity, a simple test was performed which provided definitive evidence that it is the fertilizin which is co-precipitated with serum albumin and not some hitherto undetected component of the material.

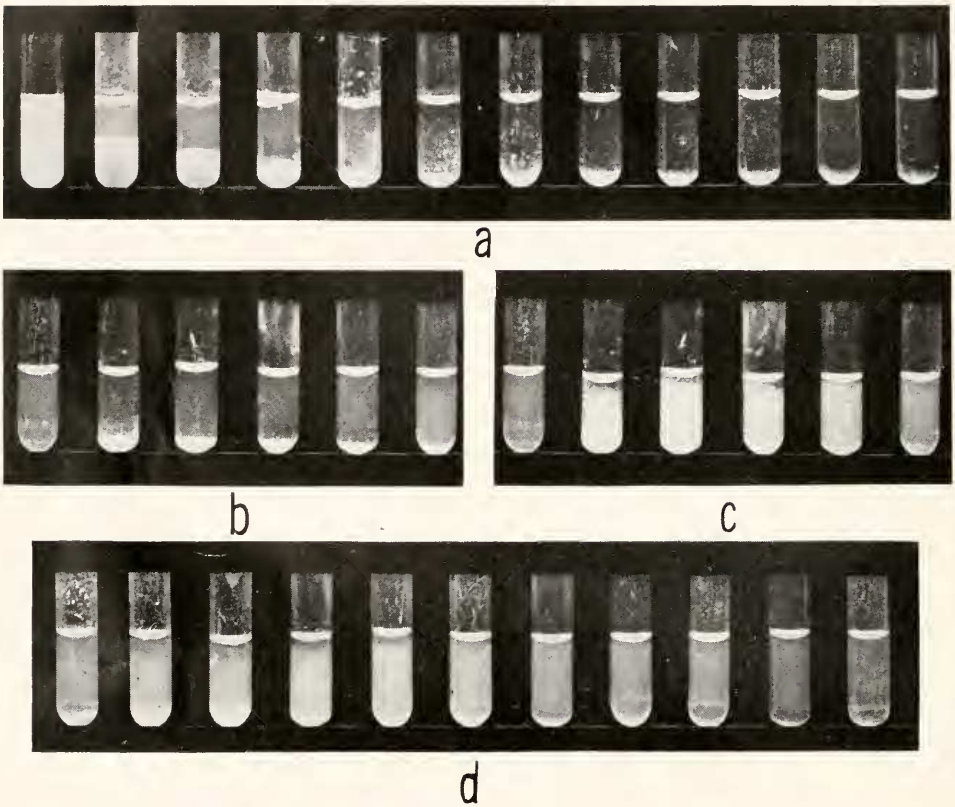

FIGURE 1. Mucin clot reactions of *Strongylocentrotus purpuratus* fertilizin and of hyaluronic acid in two-fold dilutions. **a.** Purified *S. purpuratus* fertilizin. The first tube contains fertilizin solution and serum albumin, but distilled water instead of acetic acid. The opacity of the first tube is due to the opalescence of the mixture. **b.** Crude *S. purpuratus* fertilizin. The first tube contains fertilizin solution and acetic acid but 0.9 per cent saline instead of serum albumin. c. The same as **b** with the tubes shaken prior to being photographed. **d.** Hyaluronic acid.

A solution of purified fertilizin in 3.3 per cent saline was mixed with serum albumin and the pH brought to about 3.5 with 2 N acetic acid. The resulting precipitate was thrown down by centrifugation, the supernatant withdrawn and its pH adjusted to 7. As shown in Table I, the supernatant exhibited no sperm-agglutinating activity.

TABLE I

Sperm-agglutinating activity of the supernatant and of the precipitate recovered separately after the addition of bovine serum albumin to an acidified solution of purified fertilizin, and results of control tests

Reaction mixture		Per cent of sperm-agglutinating activity*
0.5 ml. fertilizin in 3.3% saline	0.05 ml. distilled H_2O 0.2 ml. 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	Supernatant: 0 Dissolved precipitate: 100
	0.05 ml. distilled H_2O 0.2 ml. 1% bovine serum albumin in 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 0.9% saline	100
	0.05 ml. distilled H_2O 0.2 ml. 0.9% saline, acidified and neutralized	100
0.5 ml. 3.3% saline	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	0

* Sperm-agglutinating activity of fertilizin, distilled water and 0.9% saline mixture taken as 100%.

It had previously been found that the precipitate formed by the addition of serum albumin to a fertilizin solution acidified to a pH of about 3.5 dissolves completely at a pH of 5.6 or higher. After centrifugation and withdrawal of the supernatant of the material being tested, the precipitate was resuspended in 3.3 per cent NaCl solution, its pH brought to about 7, and the volume made equal to that of the original mixture. Upon testing this solution it was found, as is shown in the table, that the sperm-agglutinating activity was equal to that of the original fertilizin solution, showing that the activity was recovered quantitatively from the precipitate.

A number of control tests were made, the results of which are summarized in Table I. These showed that (1) co-precipitation of fertilizin and serum albumin does not occur in the absence of acid; (2) it does not occur in acidified solution in the absence of added protein; (3) the presence of albumin does not affect the sperm-agglutinating activity of the fertilizin; (4) the sperm-agglutinating activity is not affected by acidification and subsequent neutralization of the solution, and (5) sperm agglutination does not occur in saline solution in the absence of fertilizin, nor is a precipitate formed when albumin and acid are added to saline.

Quantitative recovery of the sperm-agglutinating activity from co-precipitated fertilizin and serum albumin, which is achieved by the simple expedient of raising the pH to 5.6 or higher, shows that the specific combining groups of the fertilizin are not irreversibly altered by its reaction with the albumin. The significance of this phenomenon cannot be evaluated at the present time, however, since essentially nothing is as yet known of the structure of the fertilizin molecule.

Titration by the mucin clot method

The method employed for titration was as follows: Two-fold serial dilutions of fertilizin in 3.3 per cent NaCl at about pH 7 are made in 10 × 75 mm. test tubes in 0.5 ml. quantities. To each tube 0.05 ml. of 2 N acetic acid is added and the contents mixed. The tubes are inclined and 0.2 ml. of 1 per cent bovine serum albumin in 0.9 per cent saline is slowly pipetted down the side of each tube; after this the tubes are carefully returned to a vertical position and in most cases a ring of precipitate forms immediately at the zone of contact between the albumin solution and the acidified fertilizin solution. The rings are easily detected at high dilutions in which a diffuse turbidity is difficult to detect and score visually. The reactions are read at once in good natural light, since the rings tend to disperse rapidly as the albumin diffuses through the mixture. The highest dilution at which a ring is observed is taken as the mucin clot titer of the preparation.

Effect of pH, albumin concentration and salt concentration

Numerous titrations with the same stock samples of purified fertilizin using the method described above have given consistently identical mucin clot titers in the course of routine testing. It was thought advisable, however, to carry out some controlled tests to determine in a more definitive manner the amount of variability in titer to be expected within a rather limited range of pH, albumin concentration, and salt concentration.

A sample of a purified fertilizin solution from eggs of *S. purpuratus* was centrifuged at 3000 r.p.m. for five minutes. A slight sediment was thrown down, the clarified supernatant was drawn off, and the pH adjusted to 7.0 with the glass electrode. To assure maximum uniformity of different samples of the supernatant, it was thoroughly mixed before removing an aliquot. Two-fold dilutions were made in 0.5 ml. quantities with 3.3 per cent NaCl solution. Serum albumin was made up in 1 per cent solutions, and the same stock solution of 2 N acetic acid was used throughout. Various amounts of the latter two solutions were added to different sets of tubes of the fertilizin dilutions. The total albumin and salt concentration in the different sets was thus altered, as noted below. The pH was measured with a Beckman glass electrode pH meter. After each titration the pH of the final mixtures was measured in the first tube, in the tube giving the end-point, and in an intermediate tube. The maximum difference in pH observed within any set was 0.26 unit. The results obtained in a series of nine titrations are given in Table II. Each of the pH values listed represents the mean of three determinations in each set. The total salt concentration is expressed as per cent NaCl. The mucin clot titers (last column of table) are the end points of visible precipitation as determined by the ring method described above.

As the data in the table show, no marked difference in mucin clot titer occurs, for the most part, as a result of the differences in pH, total salt and albumin concentrations employed. In titration 5, the observable end-point would probably have been at least one dilution higher had rings formed. The failure of rings to form is attributable to the relatively large quantity of serum albumin solution used; as noted above, in high dilutions a diffuse turbidity such as was produced in this case is more difficult to detect visually than a ring at the same dilution. It has

been found that 0.5 ml. of albumin solution is about the largest quantity practicable for obtaining consistent ring formation. Since there is always a tendency to pipette too rapidly when a large number of tests is being performed, it has proven more convenient to use 0.2 ml. of albumin solution.

The evidence presented in Table II indicates that pH is not a critical factor with respect to observed titer within the range tested. The albumin concentration is not critical, nor is the total salt concentration of the system in the range from 2.0 per cent to 3.1 per cent. Below 2.0 per cent the salt concentration may be a more important factor, as shown by the higher titer obtained in titration 6. Although the total salt concentration in titration 5 is the same as that in number 6 (1.6 per cent), the two sets are not comparable for the reason mentioned above.

TABLE II

Mean pH, total albumin concentration, total salt concentration and mucin clot titers in nine titrations using uniform samples of a homogeneous purified fertilizin preparation of S. purpuratus

Titration no.	Ml. 2 N acetic acid	Ml. 1% albumin solution	NaCl concentration of albumin solution, per cent	Mean pH	Total albumin concentration, per cent	Total NaCl concentration, per cent	Mucin clot titer
1	0.05	0.1	0.9	3.09	0.15	2.7	64
2	0.05	0.2	0.9	3.30	0.27	2.4	64
3	0.05	0.3	0.9	3.33	0.35	2.3	64
4	0.05	0.5	0.9	3.54	0.47	2.0	64
5	0.05	1.0	0.9	3.74	0.65	1.6	32*
6	0.05	0.5	0.0	3.58	0.47	1.6	128
7	0.05	0.5	3.3	3.58	0.47	3.1	64
8	0.01	0.2	0.9	3.88	0.28	2.6	64
9	0.02	0.2	0.9	3.78	0.28	2.5	64

* Scored as turbidity, not as ring.

In the next section it will be shown that the mucin clot and sperm-agglutinating titers of a fertilizin solution are both reduced after dialysis of the preparation against distilled water with consequent removal of salt (NaCl). The data to be presented indicate that the physical state of the fertilizin is reversibly modified in the absence of electrolytes, at least under certain conditions of temperature. It seems likely, therefore, that there may exist some optimum concentration of electrolytes between zero concentration and that represented by 2.0 per cent NaCl (equivalent to an ionic strength of 0.34) at which co-precipitation of fertilizin and serum albumin attains a maximum. Further analysis of the effect of salt concentration on the co-precipitation of fertilizin and serum albumin is under way and will be reported in a later communication.

In the system as employed in the routine titration procedure in the present investigation, however, the mean pH, total albumin and total salt concentration fall well within the range for each of these factors in which identical titers are obtained, using uniform samples of a homogeneous fertilizin preparation.

Mucin clot and sperm-agglutinating titrations of fertilizin in salt-free solution

A sample (I) of a stock preparation of purified fertilizin in 3.3 per cent saline was made salt-free by dialyzing against distilled water at 1° C. until it no longer formed a precipitate with $AgNO_3$. It was found that a white, flocculent material was present in the dialyzed solution, whereas no such flocculence was evident in the control in 3.3 per cent NaCl solution which was kept at 1° for the same period. A considerable reduction in both mucin clot and sperm-agglutinating titer of the salt-free sample as compared with the control was observed. In the mucin clot titration, the character of the precipitate formed in the salt-free preparation upon the addition of serum albumin in the presence of acid differed from that in the control in being of larger particle size and somewhat stringy. Difficulty was encountered in making sperm-agglutinating titrations (where the salt content of the preparation was adjusted just prior to titration to 3.3 per cent NaCl by adding an equal volume either of 6.6 per cent NaCl or of double sea water to the salt-free solution) in cases where suspended material was present. The spermatozoa clumped about the particulate matter, and it was impossible to score the dilutions satisfactorily. Where tests were made with samples of the dialyzed solution in which the amount of suspended material was visibly less than was originally present, both the mucin clot precipitation and sperm agglutination occurred in the manner characteristic of the control solutions; in these cases the two titers were also somewhat higher, although they did not necessarily equal the values obtained for the control solution.

A second sample (II) of the same stock preparation of purified fertilizin was dialyzed against distilled water until salt-free. This time the first five changes of the water used for dialysis, totaling 3 liters, were saved, combined, and lyophilized to dryness. The residue was taken up in about 10 cc. of distilled water and the resulting solution was approximately isotonic with sea water, as shown by the fact that sperm of *S. purpuratus* remained active when placed in it. This "dialysate-concentrate" was 300 times more concentrated than the original dialysate, but proved to be negative for both mucin clot formation and sperm-agglutinating activity. Sample II behaved in all respects like sample I. The results of tests with the two samples are summarized in Table III.

The evidence obtained from the present experiments indicates that at temperatures near the freezing point (1° C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes. Macroscopic aggregates may appear in a fertilizin preparation under these conditions, and there is a correlated decrease in the mucin clot and sperm-agglutinating titers. Under the influence of added salt and elevated temperature (up to 21.5° C.), either separately or combined, there occurs a correlated decrease in the amount of visible macroscopic material and increase in mucin clot and sperm-agglutinating titers to values approaching those obtained with control solutions. The negative results of tests with the "dialysate-concentrate" show that there was no actual loss of fertilizin during dialysis.

Analysis of a purified fertilizin preparation

The co-precipitation of fertilizin with protein in acidic solution in a manner analogous to the behavior of acid mucopolysaccharides suggests affinity of fertilizin with this class of substances. It has recently been claimed, moreover, that a preparation

TABLE III

Results of titrations of salt-free fertilizin preparations of S. purpuratus after various treatments

Test no.	Fertilizin preparation	Treatment after removal from dialysis bath at 1° C.	Sperm-agglutinating titration		Mucin clot titration	
			pH	Titer	pH	Titer
1	I	None			5.2	8
2	I	Salt content adjusted to 3.3% NaCl. pH adjusted	7.2	64		
3	I	Salt content adjusted to 3.3% NaCl			5.2	16
4	I	2.5 hrs. at 21.5° C.			5.2	16
5	I	2.5 hrs. at 21.5° C. pH adjusted			7.2	16
6	I	Dialyzed vs. 3.3% NaCl at 1° C. 2 hrs. at 21.5° C. pH adjusted	7.0	64	7.0	16
7	I	Dialyzed vs. 3.3% NaCl at 1° C. 24 hrs. at 8° C. pH adjusted	7.45	1024	7.45	32
8	I	Control. Not dialyzed vs. distilled water. pH adjusted	7.4	512	7.4	64
9	II	1 hr. at 21.5° C. pH adjusted			6.9	32
10	II	Salt content adjusted to 3.3% NaCl. 3 hrs. at 21.5° C. pH adjusted	6.9	512		
11	II	Salt content adjusted to 3.3% NaCl. 1 hr. at 21.5° C. pH adjusted			6.9	64
12	II	Control. Not dialyzed against distilled water. pH adjusted	7.3	1024	7.3	128

from bull testes, presumably containing the enzyme hyaluronidase, is capable of causing the jelly of intact sea-urchin eggs to swell (Ruffo and Monroy, 1946; Monroy and Ruffo, 1947). It was of interest, then, to attempt to determine the extent to which fertilizin may be chemically similar to hyaluronic acid. The few available data, which have been reviewed briefly in the introductory section, indicate that fertilizin is by no means identical with hyaluronic acid. The present investigation has shown that fertilizin differs from hyaluronic acid to a marked degree with respect to the two chief constituents of the latter substance, hexosamine and glucuronic acid.

A homogeneous sample of a purified fertilizin preparation in 3.3 per cent NaCl was prepared as described above. In the present instance the supernatant fluid was filtered through hardened filter paper. The pH of the filtrate was adjusted to 7 and

TABLE IV

Results of chemical analysis of a purified fertilizin preparation of S. purpuratus

Filtrate					Hydrolysate	
Sperm-agglutinating titer	Mucin clot titer	Dry weight, mg./ml.	Total nitrogen, per cent	Glucuronic acid	Hexosamine, per cent	α-amino acids
2048	512	8.95	4.1	none	1.6	positive Ninhydrin

mucin clot and sperm-agglutinating titers were obtained. Aliquots of the filtrate were taken for the various analyses, which included determinations of dry weight, total nitrogen, hexosamine, α-amino acids and glucuronic acid. The results of the analyses are presented in Table IV. Dry weight per ml. was calculated from the weight of material precipitated from an aliquot of the filtrate with 1.25 volumes of cold 95 per cent alcohol. According to Tyler (1948), precipitation of fertilizin is complete under these conditions. The precipitate was washed with alcohol and dried in an oven at 55° C. to constant weight. Total nitrogen was determined for duplicate samples of the dried precipitate by the micro-Kjeldahl method. Another portion of the dried material was hydrolyzed by boiling in a sealed tube with 4N HCl for eight hours. Hexosamine was determined in an aliquot of the hydrolysate by the method of Palmer, Smyth and Meyer (1937). Another portion of the hydrolysate was treated with Ninhydrin reagent for the determination of α-amino acids. For the determination of glucuronic acid the colorimetric method recently described by Dische (1947b) was employed, using a sample of the original filtrate,

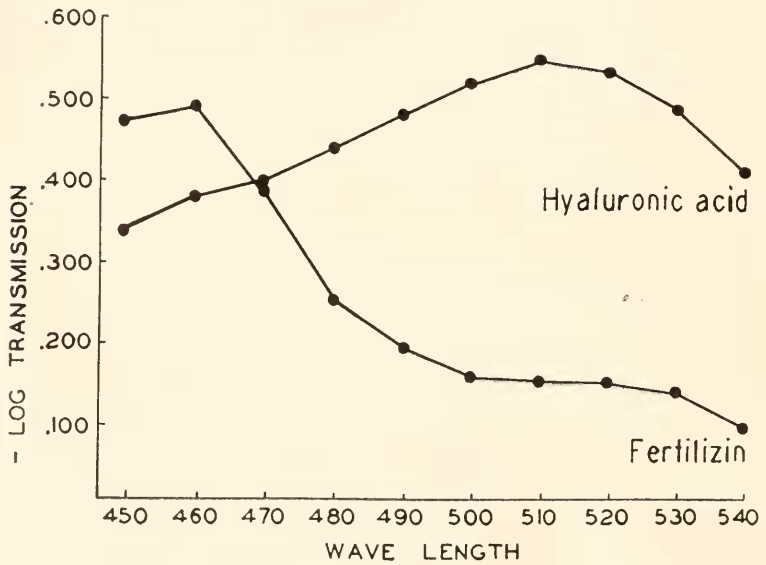

FIGURE 2. Absorption curves of reaction mixtures of mannose-thioglycolic acid-fertilizin and mannose-thioglycolic acid-hyaluronic acid. Wave lengths in mμ. Fertilizin $\Delta E_{(510-480)} = -0.097$; hyaluronic acid $\Delta E_{(510-480)} = +0.115$.

and for comparative purposes, a solution of pure potassium hyaluronate (Schering) was tested at the same time by the same method. According to Dische, the reaction of carbohydrates with -SH compounds in H_2SO_4, which differentiates between various classes of carbohydrates and individual hexoses and hexuronic acids, is highly characteristic for glucuronic acid when mannose is employed. This reaction is the basis of the test. The reaction mixture with glucuronic acid gives a typical absorption curve in the range 450–540 mμ, and it was found by Dische that the curve for hyaluronic acid is almost identical with that of glucuronic acid. In practice, accord-

ing to Dische, it is only necessary to measure the intensity of the mannose reaction at 510 and 480 mμ and subtract the second value from the first. This difference is positive for glucuronic acid and polyglucuronides, and negative for the other hexuronic acids. Figure 2 shows the absorption curves for fertilizin and for hyaluronic acid. The difference between the intensity of the mannose reaction with fertilizin at 510 and that at 480 mμ is negative, and hence it may be concluded that fertilizin does not contain glucuronic acid. This result is in agreement with previous results obtained by Tyler (unpub.) using an earlier method of Dische's (1947a). As shown in Table IV, fertilizin does contain hexosamine, but in small amount, which is in agreement with earlier results obtained by the present author using Kunitz's (1939) method. The Ninhydrin reaction was very weak but probably positive. Total nitrogen (4.1 per cent) of this material is somewhat lower than has been reported previously for *S. purpuratus* fertilizin by Tyler and Fox (1940), who found an average total nitrogen content of 5.2 per cent with crude preparations.

These data show that fertilizin differs markedly from hyaluronic acid in its chemical constitution. It is obvious, therefore, that the ability to give the mucin clot reaction does not by any means indicate close similarity between fertilizin and hyaluronic acid, even though it may be evidence that the former is related to the group of acidic mucopolysaccharides.

At the suggestion of Dr. Albert Tyler, the mucin clot titration procedure was used in conjunction with sperm-agglutinating titrations to investigate the effects of various kinds of treatment on fertilizin. In the following sections the results of parallel titrations of preparations subjected to heat and to ultra-violet irradiation are presented.

Parallel titrations with heat-treated fertilizin solutions

According to Tyler and Fox (1940), the sperm-agglutinating activity of *Strongylocentrotus purpuratus* fertilizin is rapidly destroyed at 100° C. The rate of inactivation, according to these authors, is a function of the pH, the fertilizin being most stable in the range from 4 to 7. Their data show that at pH 7.3 the agglutinin is 95–100 per cent inactivated in 20–30 minutes at 100°. In the present experiments in which *S. purpuratus* purified fertilizin solutions were used, the preparations have proven to be considerably more heat-stable than the material used by Tyler and Fox. Since the solutions employed by Tyler and Fox corresponded to crude fertilizin as defined in this paper, it may well be that the relatively purer condition of the fertilizin in the present preparations accounts for its greater stability.

Initial loss of agglutinating activity does not appear to involve complete destruction of fertilizin. At first the agglutinating fertilizin is converted into a "univalent," non-agglutinating form (cf. Tyler, 1941, Metz, 1942). It was of interest, accordingly, to test samples of heat-treated fertilizin for their univalence (inhibition) titer as well as for their sperm-agglutinating and mucin clot titers. The method employed for detecting univalent fertilizin has been briefly described in an earlier section of this paper; determination of inhibition titer consists in determining the greatest dilution in which no agglutination occurs upon the addition to the test dilutions of equal amounts of normal (untreated) fertilizin solution (Metz, 1942).

In the first experiments 1.5 ml. samples of the stock purified fertilizin solutions were placed in 13 × 100 mm. test tubes and immersed in a boiling water bath. The

exposed portions of the tubes were cooled by means of a stream of air so that heating could be continued for long periods without appreciable loss of fluid. Since sperm are quickly inactivated in even slightly hypertonic medium, the fact that the sperm remained active in the solutions that had been heated was assumed to indicate that evaporation of water from the tubes during heating was insignificant. A thermometer placed in the water bath with the tubes showed that the temperature of the bath fluctuated between 96° and 98° C.

In later experiments a quantity of fertilizin solution large enough to permit the withdrawal of a number of 1.5 ml. samples was placed in a flask with a reflux condenser attached by means of a ground glass joint. The solution was refluxed and loss of water was thus kept to a minimum. The temperature of the boiling fluid in the flask could be assumed to be about 100° C. A considerable excess of solution was used so that its concentration would not be significantly affected by the slight amount of water that failed to run back down. Before withdrawing a sample, the neck of the flask and the lower part of the condenser were cooled with cold water from a wash bottle. In all of the experiments the pH of the fertilizin solutions was adjusted with the glass electrode just before heating was begun; a control sample was allowed to stand at room temperature throughout the total time of heating. As each sample was removed from the water bath or from the reflux flask,

TABLE V

Results of parallel titrations of heat-treated, purified fertilizin preparations of S. purpuratus

Fertilizin preparation	Initial pH	Sample	Temperature, degrees Cent.	Time in hours	Final pH	Titer		
						Sperm agglutination	Mucin clot	Inhibition
I*	7.1	a	96–98	0.5	—**	256	64	—**
		b	96–98	1.0	—	64	64	—
		Control	room	1.0	—	512	64	—
II*	7.85	a	96–98	4.5	—	16	64	—
		Control	room	4.5	—	256	64	—
III*	7.4	a	96–98	3.5	—	128	256	0
		b	96–98	5.5	—	64	64	32
		Control	room	5.5	—	1024	256	—
IV***	7.5	a	100	2.0	7.5	4096	512	—
		b	100	3.0	7.2	4096	512	—
		c	100	5.0	7.1	2048	256	—
		d	100	6.0	7.1	2048	256	0
		e	100	7.5	7.1	1024–2048****	256	0
		Control	room	7.5	6.8	4096	512	—

* Individual 1.5 ml. samples heated in water bath.

** Dashes indicate not tested. Zero inhibition titer indicates tested but inhibition not detected.

*** Samples (1.5 ml.) withdrawn from refluxed solution.

**** A trace reaction probably present in the higher dilution.

it was placed immediately in the freezer. In the later experiments the pH of each heated sample was recorded before it was frozen. The titrations were performed as soon thereafter as possible. The results of the experiments are presented in Table V.

As may be seen from the table, reduction of sperm-agglutinating titer by heating is not necessarily accompanied by parallel reduction in mucin clot titer. Thus, for example, samples IIa and IIIa, heated for 4.5 and 3.5 hours respectively, showed no significant reduction in mucin clot titer although the sperm-agglutinating titer of the former was reduced to about 6 per cent and that of the latter to about 12 per cent of the original values. Sample IIIb was heated for 5.5 hours with a reduction of sperm-agglutinating titer to approximately 6 per cent of its original value. In this case the mucin clot titer was reduced to 25 per cent of the original value. Samples IVa–IVe show a more nearly parallel reduction of sperm-agglutinating and mucin clot titers than any of the others. Preparation IV was refluxed. After boiling for 7.5 hours the sperm-agglutinating titer was reduced to 25–50 per cent of the original value and the mucin clot titer was reduced to about the same per cent of the original value. Sample IIIb was the only one which gave an inhibition (univalence) titer. In the samples which were tested for inhibition but in which none was detected (IIIa, IVd, IVe), it is probable that insufficient univalent fertilizin was present in the high dilutions to permit detection. The inhibition test is unambiguous only in dilutions containing sufficient univalent fertilizin to react with most of the added sperm. In the high dilutions enough sperm remain uncombined to be agglutinated upon the addition of normal fertilizin and thus obscure the slight amount of inhibition that may be present. In the present experiments, sample IIIb was the only one in which sufficient univalent fertilizin was produced in the lower dilutions to give clear-cut evidence of inhibition. Since the inhibition titer of IIIb was 32, while the mucin clot titer was 64, it appears that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. Stronger evidence to support this view was afforded by experiments in which fertilizin was irradiated with ultra-violet light.

Parallel titrations with ultra-violet irradiated purified fertilizin preparations

Metz (1942) showed that univalent fertilizin is produced by irradiation of normal (multivalent) fertilizin by ultra-violet rays. In the present experiments ultra-violet irradiation was carried out in an apparatus consisting of glass tubing, 150 × 35 mm., fitted on the mid-section of a 15 watt General Electric "Germicidal" lamp, the diameter of which is 25 mm. The major part of the output of this lamp is concentrated in the 2537 Å wave-length band. The space between the outer wall of the lamp and the inner wall of the tubing is the irradiation chamber. The chamber and lamp assembly is mounted on a motor-driven rocker. An opening in the top of the chamber, which can be closed with a rubber stopper, permits the introduction and withdrawal of fluid. The chamber is cooled by means of a small electric fan mounted on the rocker platform; when the fan is in operation the temperature of fluid inside the chamber does not rise above 35° C. during irradiation.

In the first experiment, a purified fertilizin preparation of *S. purpuratus,* the pH of which was first adjusted to 7, was irradiated for a total of 2.5 hours. It was found, as shown in Table VI, that the sperm-agglutinating titer was reduced to

TABLE VI

Results of ultra-violet irradiation of purified fertilizin

Fertilizin preparation	Sample no.	Initial pH	Time of irradiation in hours	Final pH	Sperm agglutination titer	Mucin clot titer	Inhibition titer
I	Control	7.0	—	—	256	64	—
	1	7.0	$2\frac{1}{2}$	—	4	32	—*
II	Control	7.67	—	6.60	1024	1024	—
	1	7.67	$4\frac{1}{3}$	5.52	0	1024	128
	2	7.67	6	5.49	0	1024	128
	3	7.67	$7\frac{3}{4}$	5.49	0	512	4
	4	7.67	9	5.49	0	256	4

* Univalence present in this sample by inhibition test but titer not obtained.

about 2 per cent of the original value and the mucin clot titer was decreased to 50 per cent of the original. Tested by the inhibition method, the irradiated preparation was found to contain univalent fertilizin. The inhibition titer of this sample was not obtained. In a second experiment, a quantity of the fertilizin preparation which was found to be very heat-stable with respect to its sperm-agglutinating activity (preparation IV of the preceding section) was irradiated. The pH of the solution was first adjusted to 7.7. Small portions (1.5 ml.) were withdrawn at intervals up to nine hours; the first sample was removed after 4.3 hours of irradiation. A control sample was allowed to stand in natural light (filtered through window glass) at room temperature throughout the entire period of irradiation. Immediately upon the removal of each sample from the irradiation chamber, its pH was measured with the glass electrode, and then it was placed in the freezer. All of the samples, including the control, were stored in the freezer until the titrations could be performed. As shown in Table VI, all of the irradiated samples showed complete loss of agglutinating activity. Tested by the inhibition method, all of them were found to contain univalent fertilizin. The inhibition titers showed a progressive decrease as time of irradiation was increased. The mucin clot titers also showed a progressive decrease with increased time of irradiation. After nine hours the mucin clot titer was reduced to 25 per cent of its original value, and the inhibition titer was reduced to about 6 per cent of the value found after 4.3 hours of irradiation.

The results of these experiments demonstrate conclusively that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. They also show that ultra-violet irradiation is a more effective agent than heat in converting multivalent, purified fertilizin to the univalent condition. The progressive decrease in inhibition titer found in the second experiment indicates that degradation of the fertilizin by ultra-violet light proceeds beyond the stage in which it exhibits univalence.

DISCUSSION

In general it may be said that the mucin clot titer of untreated fertilizin preparations parallels their sperm-agglutinating activity. Sperm agglutination is usually detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

Destruction of the sperm-agglutinating activity of fertilizin is not necessarily accompanied by a reduction of mucin clot titer. Conversely, however, it is clear that fertilizin which has been subjected to treatment that causes a reduction in mucin clot titer, for example heating or irradiation by ultra-violet light for extended periods, will invariably show at least a parallel decrease in sperm-agglutinating activity. It has been shown in the experiments with ultra-violet irradiation that the capacity of the fertilizin to agglutinate sperm may be completely destroyed with but little, if any, loss of its ability to give the mucin clot reaction. The evidence shows that when the agglutinating (multivalent) form is degraded to the non-agglutinating (univalent) form, the latter continues to co-precipitate with protein in the mucin clot reaction. If a preparation in which all of the fertilizin has been made univalent is subjected to continued irradiation by ultra-violet light, a progressive decrease in both mucin clot and inhibition titers occurs.

The phenomenon of sperm agglutination by fertlizin has been interpreted by Tyler (1941, 1942, 1947, 1948) as an antigen-antibody type of reaction in which complementary combining groups of a substance (antifertilizin) on the surface of the sperm cells unite in "lock and key" fashion with the combining groups of fertilizin. Where a number of such combining groups are available on the surface of the fertilizin molecule, agglutination occurs as the result of the building up of a lattice, as postulated for analogous immunological reactions by Heidelberger (1938) and Marrack (1938). The formation of univalent fertilizin is brought about by various agents—e.g. heat, ultra-violet light, x-rays—which, according to Tyler (1941), split the molecule into fragments, each of which contains a single combining group. These fragments are still of large size, since they are non-dialyzable (Tyler, 1941). They are also capable of co-precipitating with protein in the presence of acid, giving the mucin clot reaction.

The ability to give the mucin clot reaction is, at least in the case of hyaluronic acid, presumably a function of the degree of polymerization of the molecule (Meyer, 1947). Depolymerized molecules are incapable of giving the reaction. Although fertilizin has been shown to be very different from hyaluronic acid in its chemical composition, the fact that it co-precipitates with protein in acid solution in an analogous manner suggests that it may be similar in its physical structure. Thus, fertilizin may normally exist in a polymerized condition. Sperm-agglutinating activity may, then, accompany a range of polymer size, and the univalent condition may represent a state of polymerization with which but a single combining element is associated. Degradation of multivalent fertilizin to the univalent form would then entail a progressive splitting off of relatively stable univalent units. The evidence from the experiments with ultra-violet irradiation indicates that the univalent form is in fact the more stable, since complete conversion to univalence was observed after 4.3 hours of irradiation, whereas even after nine hours both the inhibition and mucin clot titers retained significant values.

Summary

1. Preparations of fertilizin of three species of sea-urchin have been found to give a mucin clot reaction similar to that given by hyaluronic acid. Upon the addition of bovine serum albumin to an acidified solution of fertilizin, a precipitate forms which dissolves at a pH of 5.6 or higher. All of the sperm-agglutinating activity accom-

panies the precipitate and it is recovered quantitatively when the precipitate is dissolved.

2. A method for the determination of mucin clot titer of fertilizin is described.

3. At temperatures near the freezing point (1° C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes by dialysis. Macroscopic aggregates appear, accompanied by a parallel decrease in mucin clot and sperm-agglutinating titers. Disappearance of the aggregates is accompanied by an increase in both titers.

4. Chemical analysis of fertilizin shows that it contains no glucuronic acid, about 2 per cent hexosamine and amino acids. Fertilizin, therefore, differs greatly from hyaluronic acid, but its ability to give the mucin clot reaction suggests an affinity with the class of mucopolysaccharides.

5. In general, mucin clot titer parallels sperm-agglutinating titer of the same untreated fertilizin preparation, although sperm agglutination is detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

6. Parallel mucin clot and sperm-agglutinating titrations were made with fertilizin preparations in untreated condition and after exposure to heat and to ultra-violet irradiation. The purified preparations used in these experiments proved to be exceptionally heat-stable; irradiation by ultra-violet light was found to be a more effective treatment in degrading the material.

7. Destruction of the sperm-agglutinating activity of fertilizin by heat and by ultra-violet irradiation does not necessarily cause a parallel decrease in mucin clot titer. The mucin clot reaction continues to be given by preparations in which all of the fertilizin has been converted from the normal, agglutinating condition to the non-agglutinating, "univalent" form. Continued irradiation of the univalent fertilizin is accompanied by a progressive decrease in both inhibition and mucin clot titer.

8. It is suggested that fertilizin may normally exist in a polymerized condition and that the non-agglutinating, "univalent" condition may represent a relatively more stable lower polymer of the native, agglutinating form.

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